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Minireview

mRNA quality control: An ancient machinery recognizes and degrades mRNAs with nonsense codons

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Abstract Nonsense-mediated mRNA decay (NMD) is an mRNA surveillance pathway which ensures the rapid degradation of mRNAs containing premature translation termination codons (PTCs or nonsense codons), thereby preventing the accumulation of truncated and potentially harmful proteins. In this way, the NMD pathway contributes to suppressing or exacerbating the clinical manifestations of specific human genetic disorders. Studies in model organisms have led to the identification of the effectors of the NMD pathway, and illuminated the mechanisms by which premature stops are discriminated from natural stops, so that only the former trigger rapid mRNA degradation. These studies are providing important insights that will aid the development of new treatments for at least some human genetic diseases.

Keywords: EJC; mRNA decay; NMD; PABP

1. Introduction

The eukaryotic gene expression pathway involves a number of interlinked steps from transcription of the genetic material to protein synthesis, with messenger RNAs (mRNAs) being the key intermediates in this process. The mRNA precursor is transcribed from DNA, processed by removal of introns and addition of the cap structure and the poly(A) tail, and the mature mRNA is then exported to the cytoplasm where it is translated to protein and finally degraded. It is now established that most, if not all, of the steps of the gene expression pathway can be regulated, allowing the expression of individual genes to be controlled.

While the complexity of eukaryotic gene expression allows for the production of proteins to be controlled at many levels, it also makes the process vulnerable to errors, which may occur at any step. Eukaryotic cells have evolved elaborate mRNA quality control mechanisms that ensure the fidelity of gene expression by detecting and degrading aberrant transcripts [1]. These quality control mechanisms (or mRNA surveillance mechanisms) operate in both the nucleus and the cytoplasm. For example, improperly processed mRNAs are degraded by mRNA surveillance mechanisms in the nucleus before they are exported [1]. In the cytoplasm, quality control mechanisms assess the translatability of the mRNA and degrade any that lack translation termination codons (non-stop-mediated mRNA decay, NSD) or that have premature translation termination codons (nonsense-mediated mRNA decay, NMD), thereby preventing the accumulation of potentially toxic protein fragments [1].

mRNA surveillance pathways act not only on aberrant mRNAs, but also regulate the expression of naturally occurring transcripts having features recognized by the surveillance machineries. In this way, surveillance pathways also contribute to the post-transcriptional regulation of gene expression [2].

One of the best characterized mRNA quality control mechanisms in eukaryotes is the nonsense-mediated mRNA decay (NMD) pathway, which rids the cell of mRNAs with premature translation termination codons (PTCs or nonsense codons) [2–5]. In this review, we discuss new insights into the molecular mechanisms of NMD gained from studies in model organisms. These studies reveal that whilst the NMD machinery is conserved, different species have evolved different mechanisms of recognizing and degrading aberrant transcripts. The mechanism by which nonsense mRNAs are recognized impacts on the effectors as well as on the endogenous targets of this pathway.

2. Nonsense-mediated mRNA decay: a summary

Nonsense-mediated mRNA decay is triggered by premature translation termination, which leads to the assembly on the mRNA of what is known as a 'surveillance complex'. The role of the surveillance complex is to couple a premature translation termination event to mRNA degradation by interacting with both eukaryotic translation termination factors (i.e. eukaryotic release factors 1 and 3; eRF1 and eRF3), and with the general cellular mRNA decay machinery, thereby accelerating degradation of the aberrant mRNA [2–5].

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Abbreviations: DSE, downstream sequence element; EJC, exon junction complex; NMD, nonsense-mediated mRNA decay; PTC, premature translation termination codon; SMG, suppressor with morphological effect on genitalia; UPF, up-frameshift; UTR, untranslated regions

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The surveillance complex consists of the conserved NMD effectors, which include three yeast (UPF1, UPF2 and UPF3: up-frameshifts 1–3) and seven *Caenorhabditis elegans* proteins (SMG1–7, suppressor with morphological effect on genitalia 1–7). These effectors were identified in genetic screens in *Saccharomyces cerevisiae* and *C. elegans*, or by homology searches in other organisms [2–5]. They play an essential role in NMD in all organisms in which this process has been investigated (Table 1). The UPF1, UPF2 and UPF3 proteins (also known as SMG-2, SMG-3 and SMG-4 in *C. elegans*) are conserved in eukaryotes including *S. cerevisiae*, *C. elegans*, *Drosophila melanogaster*, *Homo sapiens*, as well as *Arabidopsis thaliana* [2–5]. There are two *UPF3* paralogue genes in vertebrates (*UPF3a* and *UPF3b* also known as *UPF3* and *UPF3x*). These proteins are unlikely to be redundant, because depletion of human UPF3b, but not of UPF3a, abolishes NMD of some mRNAs containing nonsense codons [6,7].

The additional NMD effectors (SMG1, SMG5, SMG6 and SMG7) are conserved in most multicellular organisms, but SMG7 has no clear orthologue in *D. melanogaster*. SMG5, SMG6 and SMG7 are homologous to the *S. cerevisiae* telomerase associated protein Est1p and the related protein Ebs1p, but we do not know whether these proteins play a role in NMD [8–10].

UPF1 is an RNA helicase whose activity is regulated by cycles of phosphorylation and dephosphorylation, and this requires the additional NMD effectors (Fig. 1). Phosphorylation of UPF1 requires UPF2 and UPF3, and is catalyzed by SMG1, a protein kinase related to phosphoinositide-3-kinases [11–14]. Dephosphorylation of UPF1 is mediated by SMG5, SMG6 and SMG7, three related proteins which act as adaptors between phosphorylated UPF1 and protein phosphatase 2A (Fig. 1) [15–19]. SMG5–7 bind phosphorylated UPF1 through specific domains that adopt a 14-3-3-like fold [8]. Despite the absence of clear orthologues of SMG1 and SMG5–7 in *S. cerevisiae*, both UPF1 and UPF2 are phosphorylated in this organism [20]. However, the identity of the kinase(s) responsible for these phosphorylations and how dephosphorylation occurs, remain to be established.

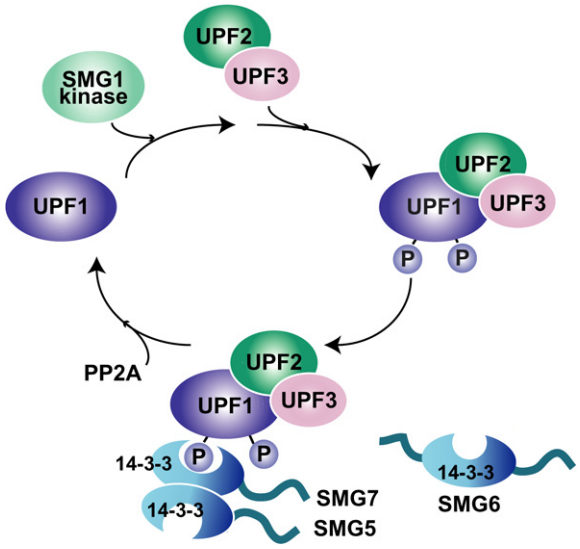
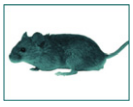


Fig. 1. UPF1 is a conserved RNA helicase essential for NMD. UPF1 is phosphorylated at multiple serine residues. Phosphorylation of UPF1 is catalyzed by SMG1, a phosphoinositide-3-kinase-related protein kinase and requires UPF2 and UPF3 [11–14,19]. The dephosphorylation of UPF1 is mediated by SMG5, SMG6 and SMG7, three similar, but not redundant, proteins. These proteins bind phosphorylated UPF1 through specific domains, which adopt a 14-3-3-like fold [8], and trigger UPF1 dephosphorylation by recruiting phosphatase 2A (PP2A) [15–18].

The precise mechanism by which NMD effectors assemble on mRNAs that terminate translation prematurely is not completely understood and differs between species (Fig. 2) [3–5,21]. It is generally accepted that UPF1 is recruited to mRNAs by ribosomes terminating translation prematurely. This recruit-

Table 1
NMD effectors in the organism for which NMD has been investigated are shown as well as the phenotypes resulting from the inhibition of the pathway by gene knockouts (KO) or knockdowns (KD)

Organism	 Yeast (*Saccharomyces cerevisiae*)	 Nematodes (*Caenorhabditis elegans*)	 Fruitfly (*Drosophila melanogaster*)	 Mammals (*Mus musculus*)	 Plant (*Arabidopsis thaliana*)
Effectors	Upf1 Upf2 Upf3	SMG-2(UPF1) SMG-3(UPF2) SMG-4(UPF3) SMG-1 SMG-5 SMG-6 SMG-7	UPF1 UPF2 UPF3 SMG1 SMG5 SMG6	UPF1(RENT1) UPF2 UPF3a/b SMG1 SMG5 SMG6 SMG7	UPF1(IBA1) UPF2 UPF3 nd nd nd nd
Phenotype	Not essential [5]	Viable worms with morphological effects on genitalia [11,15,23,40]	UPF1 and UPF2 required for larval development and cell proliferation [43,57]	UPF1JKO: embryonic lethal required for cell cycle progression UPF3 KD: effects on cell proliferation [7,50,58]	UPF1 and UPF3: required for plant development and survival [47]

Whether plants have orthologues of SMG1 and SMG5–7 has not been determined (nd).

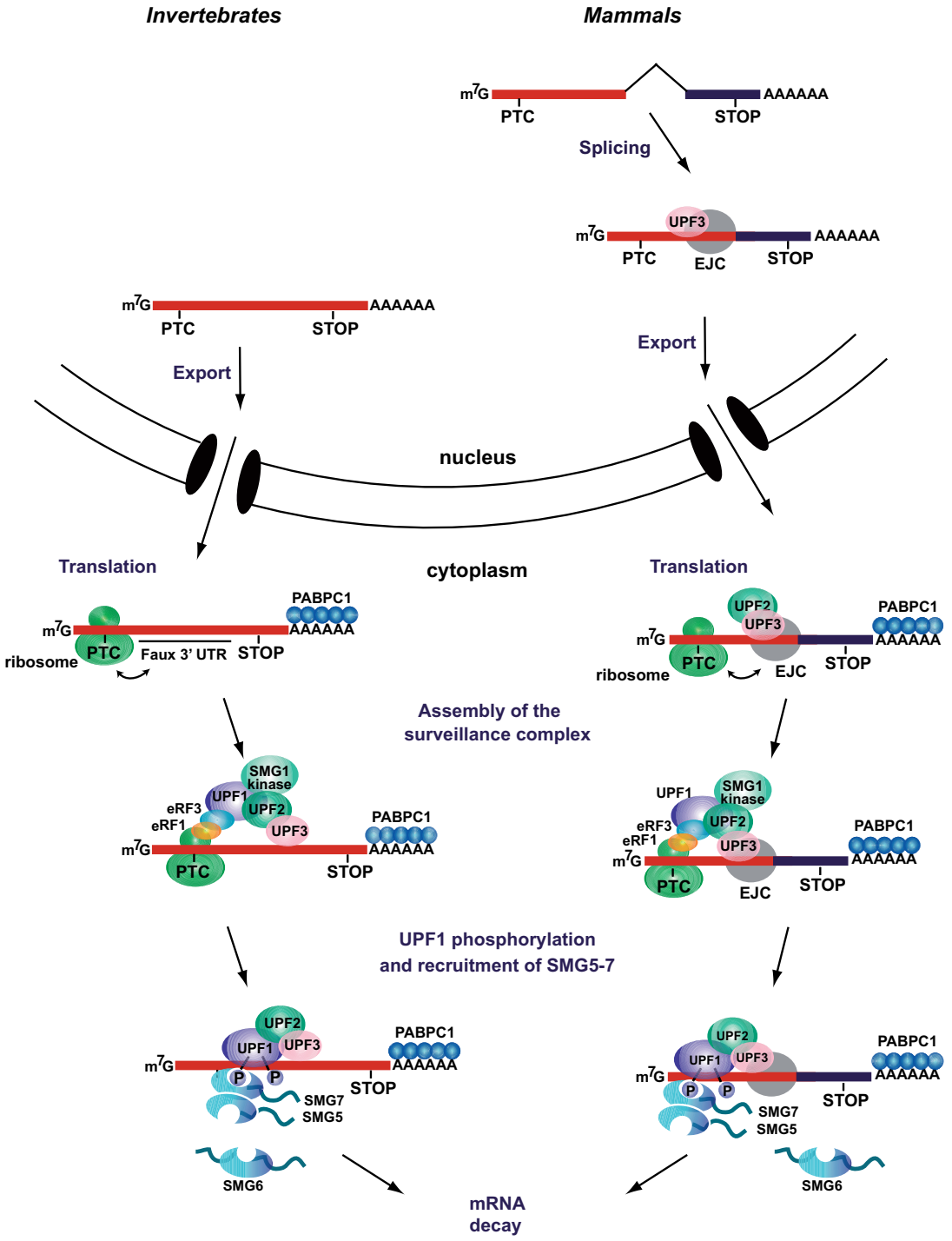


Fig. 2. The NMD pathway. A premature translation termination codon (PTC) is defined relative to the position of a downstream *cis*-acting signal that varies across species [3–5]. In mammals, the *cis*-acting signal is an exon–exon junction marked by a group of proteins that constitute the exon junction complex (EJC). The EJC is a multiprotein complex deposited at exon–exon boundaries during splicing (grey circle). A translation termination event upstream of an EJC leads to the recruitment of UPF1 and SMG1 by the eukaryotic translation release factors eRF1 and eRF3. UPF1 then interacts with UPF2, UPF3, and additional EJC proteins bound to a downstream EJC. These interactions result in the assembly of the surveillance complex, the phosphorylation of UPF1 by SMG1 and ultimately mRNA degradation. In *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* the *cis*-acting signal is a *faux* 3′ UTR [5,25,38–40]. Natural termination codons (STOP), in contrast to PTCs, are flanked by proper 3′ UTRs marked by a specific protein or set of proteins, including the cytoplasmic poly(A)-binding protein (PABPC1), which is thought to increase the efficiency of translation termination. Premature termination is inefficient because the PTC lacks proximal PABPC1 and/or other factors associated with a natural 3′ UTR. In this case, the NMD factors UPF1–3 are recruited via interactions with the eRFs. Again, this results in the assembly of the surveillance complex, phosphorylation of UPF1 and degradation of the nonsense mRNA.

ment is mediated by the translation release factors eRF1 and eRF3, which form a complex with UPF1 and SMG1 [19,22]. Subsequently, UPF1 interacts with UPF2 and UPF3 forming the core of the surveillance complex. In metazoans, formation

of the UPF1:UPF2:UPF3 complex on the mRNA triggers UPF1 phosphorylation by SMG1 [11–14,19,21,23]. Phosphorylated UPF1 is then recognized by the SMG5–7 proteins [8,15,16]. These proteins are thought to provide a molecular link between the core of the surveillance complex and the general cellular mRNA decay enzymes [24], which degrade the mRNA exonucleolytically from the two ends of the transcript. Additionally, SMG5–7 recruit protein phosphatase 2A resulting in UPF1 dephosphorylation [15–18]. This is likely to play a role in the recycling of NMD effectors for a new round of NMD.

In *S. cerevisiae* the lack of SMG5–7 orthologues suggests that the decay enzymes are likely to be recruited to nonsense mRNAs via direct interactions with the UPF1:UPF2:UPF3 trimeric complex. In *D. melanogaster*, the absence of a clear SMG7 orthologue together with the observation that decay of nonsense mRNAs is initiated by endonucleolytic cleavage near the PTC, suggest that once assembled, the surveillance complex recruits a specific NMD endonuclease [25,26]. Finally, as discussed below, alternative NMD pathways that are independent of either UPF2 or UPF3 have been described in human cells [7,27], suggesting that during evolution additional proteins may have acquired similar functions.

3. Mechanism of premature stop codon recognition in mammals

The initial step in the NMD pathway is the recognition of a stop codon as premature. This raises the question of how a premature stop codon is discriminated from a natural stop, so that only the former triggers mRNA decay. Insights into this question have been provided by the observation that the ability of a nonsense codon to elicit mRNA degradation (i.e. NMD) depends on its location relative to downstream sequence elements and associated proteins [3–5].

In mammals, these downstream sequences are represented by exon–exon boundaries (Fig. 2). Indeed, natural stops are typically located within terminal exons, whereas destabilizing nonsense codons lie more than 50–55 nucleotides upstream of the last exon–exon boundary [3–5,28]. Exon–exon boundaries are marked by the exon junction complex (EJC), a multiprotein complex deposited during splicing 20–24 nucleotides upstream of a splice junction [29]. That the EJC provides positional information to discriminate premature from natural stops is supported by the observation that at least five EJC components are required for NMD in human cells. These include the protein RNPS1, the Y14:MAGOH heterodimer and additional binding-partners such as the protein Barentsz and the RNA helicase eIF4AIII [30–35]. Moreover, UPF3b interacts with EJC proteins and is considered to be a peripheral component of the EJC [29,36].

The association of UPF3b with the EJC in mammalian cells together with its nuclear localization suggest that UPF3b is loaded onto mRNAs within the nucleus and recruits UPF2 (which is cytoplasmic) following the export of the mRNA to the cytoplasm [29,36,37]. When translating ribosomes encounter a stop codon upstream of an EJC, the recruitment of UPF1 by the release factors eRF1 and eRF3 [19,21], enables the interaction of UPF1 with the UPF2 and UPF3 proteins bound to the downstream EJC, and thus to the assembly of the surveillance complex, phosphorylation of UPF1 by SMG1 and ultimately to mRNA degradation (Fig. 2) [19,21]. As mentioned above, it is possible that some EJC proteins can functionally substitute for UPF2 or UPF3, because NMD pathways independent of either of these proteins have been described [7,27].

4. Mechanism of premature stop codon recognition in invertebrates

Recent studies in *S. cerevisiae*, *D. melanogaster* and *C. elegans* have shown that in these organisms PTC-recognition occurs independently of splicing, and thus of exon–exon boundaries [25,38–40]. Consequently, EJC components are not required for NMD in *C. elegans* and *D. melanogaster*, in spite that they are conserved in these organisms [25,40].

Several models have been proposed to explain how PTCs are discriminate from normal stops in invertebrates [3–5]. One model proposes that mRNAs harbour loosely defined downstream sequence elements (DSEs) with a function analogous to that of mammalian exon junctions [3–5]. Alternative models suggest that a generic feature of the mRNA, such as the poly(A)-tail or a mark deposited during the cleavage and polyadenylation reaction could provide positional information to discriminate premature from natural stop codons [3–5,41,42].

A third model, the 'faux 3′ UTR model', proposes that premature translation termination is intrinsically aberrant because the stop codon is not in the appropriate context [5,38]. Indeed, experiments in *S. cerevisiae* have shown that translation termination is aberrant at premature stop codons, and that prematurely terminating ribosomes are not released efficiently. Flanking the nonsense codon with a normal 3′ UTR abolishes this effect [5,38].

According to the 'faux 3′ UTR model', natural 3′ UTRs are marked by a specific set of proteins that influence translation termination. Termination is efficient at natural stops because terminating ribosomes are able to interact with these 3′ UTR-bound proteins. In contrast, translation termination would be impaired or too slow at premature stops, because these codons are flanked by a 'faux 3′ UTR' and terminating ribosomes cannot establish the appropriate interactions (Fig. 2). In this case, the surveillance complex is assembled, leading to the rapid degradation of the mRNA [5,38].

A protein that characterizes natural 3′ UTRs is the poly(A)-binding protein (yeast Pabp1 or metazoan cytoplasmic poly(A)-binding protein PABPC1, Fig. 2). Tethering of Pab1p or PABPC1 downstream of a PTC leads to efficient translation termination and NMD suppression in *S. cerevisiae* or *D. melanogaster*, respectively [5,38,39]. This suggests that the tethered protein is sufficient to mimic a normal 3′ UTR. This observation also indicates that the role of Pab1p (or PABPC1) in PTC-definition is independent of 3′-end mRNA cleavage and polyadenylation.

How can Pabp1 (or PABPC1)-binding suppress NMD? Pabp1 (or PABPC1) interacts with eRF3 [5,19,22,38], therefore tethering of Pabp1 downstream of a PTC is likely to increase the efficiency of translation termination and ribosome release, thus abolishing NMD. In agreement with this model, tethering

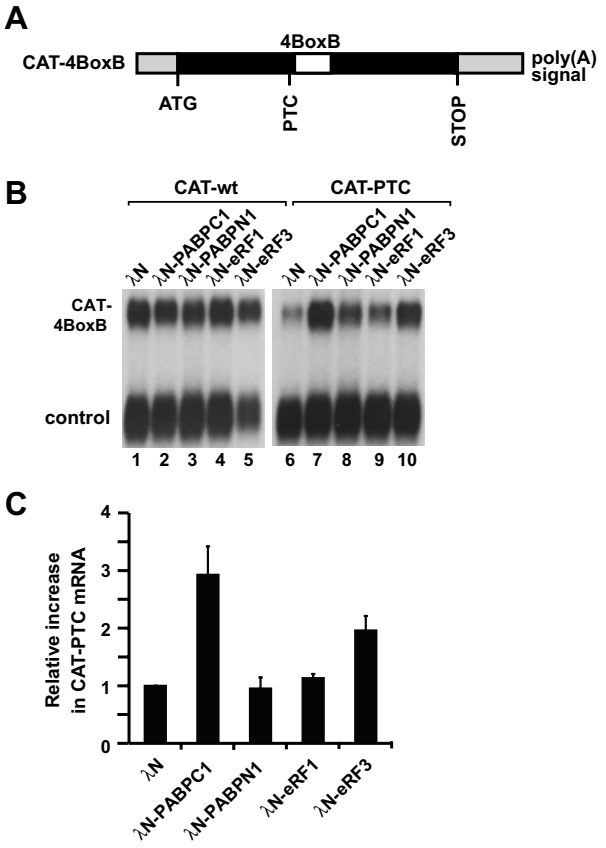


Fig. 3. Tethered eRF3 suppresses NMD. (A) Schematic representation of the tethering reporter. This assay is based on the high affinity interaction between the RNA-binding peptide derived from the bacteriophage-λN protein (λN-peptide) and an RNA hairpin known as BoxB element. Proteins of interest fused to the λN-peptide are coexpressed with a CAT reporter mRNA in which four BoxB (4BoxB) elements are inserted in frame at the same position in a wild-type reporter (CAT-wt) or a PTC-containing reporter (CAT-PTC), downstream of a PTC. Black boxes: CAT gene; gray boxes 5′ and 3′ UTRs derived from the vector. White box: 4BoxB elements. (B,C) S2 cells were transfected with the CAT-4BoxB reporters with or without PTC, a transfection control and plasmids encoding the λN-peptide fusions of the proteins indicated above the lanes. Total RNA samples were analyzed by Northern blot (B). The levels of the CAT mRNA reporters were normalized to the levels of the transfection control in three independent experiments. The normalized values obtained for CAT-PTC were divided by those obtained for CAT-wt for each protein (to compensate for potential unspecific effects of the overexpressions). This ratio was set to 1 in cells expressing the λN-peptide alone. Mean values ± S.D. of three independent experiments are shown (C).

of eRF3 downstream of a PTC also abolishes NMD both in *S. cerevisiae* and *D. melanogaster*, albeit less effectively than does tethered Pabp1 (or PABPC1; Fig. 3) [5,38]. Pabp1 may also recruit other translational regulators that stimulate translation termination or proteins that prevent NMD when translation terminates at the physiological stop codon.

The role of PABPC1 in NMD goes beyond providing positional information for PTC-definition because depletion of PABPC1 from *D. melanogaster* cells inhibits NMD under conditions in which translation efficiency is not affected [39]. A possible additional role for PABPC1 could be that of facilitating the recruitment of the NMD machinery. However, stable, RNA-independent interactions between PABPC1 and NMD factors have not yet been reported, suggesting that if these interactions occur they may be indirect or transient [39].

5. Potential impact of NMD in the evolution of eukaryotic 3′ UTRs

The observation that Pabp1 (or PABPC1)-binding downstream of a PTC converts it into a normal stop codon indicates that what defines a normal stop is the proximity to the poly(A) tail (i.e. the Pabp1 or PABPC1-binding site). If this were the case, increasing the distance of natural stop codon to the poly(A) tail should convert it into a PTC. This is indeed the case: aberrant transcripts with exceptionally long 3′ UTRs (due to errors in 3′ end-processing) are regulated by NMD in *S. cerevisiae* [41]. Similarly, in *D. melanogaster* natural stops can be made to trigger NMD by increasing 3′ UTR length [39].

Consistent with the possibility that stop codons are defined as premature or natural depending on the distance to the poly(A) tail, most *S. cerevisiae* 3′ UTRs are homogeneous in length (ca. 100nt) [5]. However, in contrast to yeast, in multicellular organisms 3′ UTR length ranges from a few to several thousand nucleotides, and a large proportion of naturally occurring transcripts with exceptionally long 3′ UTRs are not NMD substrates [39,43]. This suggests that some of these 3′ UTRs may have evolved features to avoid NMD.

There are several mechanisms by which mRNAs with long 3′ UTRs may escape NMD. For example, inherently unstable mRNAs are insensitive to a further acceleration of their decay by NMD [39]. Alternatively, long 3′ UTRs may have sequence elements that are folded via RNA–RNA or RNA–protein interactions into structures that bring PABPC1 into close proximity with the natural stop. In this case, proximity to PABPC1 will not be directly correlated to the length in nucleotides between the natural stop and the poly(A) tail.

An alternative mechanism to confer resistance to NMD may involve the presence of mRNA stabilizing elements that prevent degradation directly. Sequence elements that antagonize NMD have been reported in viral and *S. cerevisiae* transcripts. A sequence element that stabilizes unspliced Rous sarcoma virus RNAs also inhibits NMD when located downstream of a PTC [44]. The *S. cerevisiae PGK1*, *GCN4* and *YAP1* mRNAs have sequence elements that prevent NMD in a heterologous context when positioned downstream of a PTC [45].

The effects of the *S. cerevisiae* stabilizing elements are mediated by the RNA-binding protein PUB1 [45]. It is unclear whether PUB1 prevents decay or increases the efficiency of translation termination at nonsense codons located upstream of its binding site, but these two modes of action could be envisaged for different RNA-binding proteins. Immunity or susceptibility to NMD could then be regulated if these proteins were to be expressed under specific physiological conditions or in a tissue-specific manner. In this way, NMD could contribute to changes in gene expression in response to altered physiological conditions or in specific tissues.

The existence of a mechanism of PTC-recognition based on the position of the stop relative to the poly(A) tail, together with the observation that natural stops are redefined as premature by increasing 3′ UTR length suggests that NMD is likely

to present a selective constraint on the evolution of eukaryotic 3′ UTRs, so that the increase in 3′ UTR lengths to accommodate regulatory elements and miRNA-binding sites in multicellular organisms must have co-evolved with the acquisition of features to suppress NMD.

6. Evolution of the NMD pathway

The comparison of the NMD pathway in mammalian cells and invertebrates suggests that the mechanism by which nonsense codons are defined changed during evolution from a more ancestral PABPC1-dependent mode to a predominantly EJC-dependent mode [2]. However, recent studies in plants have challenged this view. Indeed in *Nicotiana tabacum* leaves, both long 3′ UTRs and exon–exon boundaries elicit efficient NMD when inserted downstream of a stop codon, indicating that these two mechanisms were present in the common ancestors of extant eukaryotes [46,47].

In agreement with this, examples in which PTCs are recognized in mammals in the absence of downstream EJCs have been described ([48] and references therein), although steady-state mRNA levels are in general only moderately affected in these cases. This raises the question of whether the two mechanisms of NMD (i.e. splicing-dependent and PABPC1-dependent) still coexist in mammals and perhaps in other eukaryotes.

It has been argued that the major mechanism of PTC-recognition in eukaryotes is PABPC1-dependent and that the role of EJC components is to enhance NMD [48]. However, the observation that exon–exon boundaries elicit NMD when located downstream of stop codons in mammals and *N. tabacum*, but not in *S. cerevisiae*, *C. elegans* and *D. melanogaster*, points to the existence of two independent mechanisms of PTC-recognition (i.e. splicing dependent and splicing-independent). In agreement with this, available evidence indicates that PABPC1 is dispensable for NMD in human cells, provided that the PTC is located upstream of an exon–exon boundary [49].

One consequence of the splicing-dependent mechanism for PTC-definition in mammals is the requirement of EJC components for NMD. These include the human proteins Y14, MAGOH, eIF4AIII, Barentsz and RNPS1 [30–35]. Their orthologues in *C. elegans* and *D. melanogaster* are not required for NMD [25,40]. Moreover, there are no clear orthologues of these proteins in *S. cerevisiae*. In this context, it would be of interest to determine whether these proteins are required for NMD in plants or whether EJC components have been co-opted only by the mammalian NMD machinery.

The splicing-dependent mechanism for PTC-recognition has also important consequences for the evolution of the NMD pathway. Indeed, the existence of an intermediate step in the assembly of the surveillance complex in which NMD effectors interact with EJC components (Fig. 2), may have created the opportunity for EJC proteins to functionally substitute for UPF2 or UPF3 as reported by Gehring et al. and Chan et al. [7,27]. In turn, the release of functional constraints on NMD effectors may have facilitated the acquisition of additional functions. Indeed, mammalian UPF1, SMG1 and SMG5–7 have been implicated in cellular processes distinct from NMD [2,9,10,50–52].

7. Functional diversification of the effectors of NMD

In human cells, Mendell et al. reported that UPF1 targets are also regulated by UPF2 [53]. In contrast, Gehring et al. identified endogenous transcripts that are regulated by UPF1 but remain unaffected in cells depleted of UPF2 [27]. Similarly, Chan et al. identified NMD substrates that are dependent on UPF1 and UPF2 but not UPF3 [7]. Based on these and additional observations, Gehring et al. and Chan et al. proposed the existence of alternative branches of the NMD pathway in human cells, namely a UPF2-independent and a UPF3-independent pathway [7,27], which is in agreement with the analysis of expression profiles in human cells depleted of UPF2 or UPF3 [2,7,27]. Indeed, UPF2 or UPF3 regulate only a subset of UPF1 targets and vice versa [2,7,27].

The lack of genome-wide information on genes regulated by the additional components of the NMD machinery in human cells leaves open the question of whether transcripts regulated exclusively by UPF1, UPF2 or UPF3 represent NMD targets, or rather reflect transcripts regulated by these proteins by mechanisms distinct from NMD, providing evidence for a functional diversification of these NMD effectors. That UPF1, UPF2 and UPF3 might have partially non-overlapping functions is further supported by the observation that human cells depleted of UPF1 are arrested early in S-phase, depletion of UPF2 has no apparent phenotype, and depletion of UPF3b affects cell proliferation (Table 1) [7,50].

Evidence that UPF1 does indeed have additional roles already exists. UPF1 can be recruited to the 3′ UTR of specific transcripts via interactions with Staufen1 (a double-stranded RNA-binding protein), and promotes mRNA degradation by a mechanism not requiring UPF2 or UPF3 [51]. Moreover, UPF1 is implicated in the degradation of histone mRNAs. In this case, UPF1 is recruited to the conserved stem–loop structure present at the 3′-end of histone mRNAs via interactions with the stem–loop-binding protein (SLBP) [52]. These observations raise the possibility that other RNA-binding proteins might act in a similar way.

In contrast to the situation in human cells, gene expression profiles in *S. cerevisiae* and *D. melanogaster* cells depleted of NMD effectors are strikingly similar, making it unlikely that these proteins have acquire additional roles in mRNA metabolism in these organisms [2,43,54]. However, it remains possible that NMD effectors have acquired specialized functions that do not affect steady-state mRNA levels, and thus that are not revealed by microarray analysis.

8. NMD regulates a significant fraction of the transcriptome

Gene expression profiling of *S. cerevisiae*, *D. melanogaster* and human cells depleted of essential NMD factors has allowed the identification of a cohort of transcripts regulated by NMD. These studies revealed that NMD regulates the expression of approximately 3–10% of the transcriptome [2,43,53,54].

As expected, aberrant mRNAs with PTCs are found amongst NMD targets. These include inefficiently spliced mRNAs that are exported to the cytoplasm with intronic sequences, alternatively spliced mRNA isoforms with PTCs, and transcripts derived from pseudogenes and transposable

elements [2,43,53,54]. In mammals, mRNAs transcribed from unproductively rearranged immunoglobulin and T-cell receptor genes also represent an important physiological class of NMD substrates [2]. Indeed, about two thirds of such recombination events result in frame-shifted genes whose transcripts are degraded by NMD [2–5,7].

In addition to PTC-containing mRNAs, a large set of 'error-free' transcripts is also regulated by NMD [2,43,53,54]. These 'error free' NMD targets represent a heterogeneous group of mRNAs and include (1) mRNAs with upstream open reading frames (uORFs) in the 5′ untranslated region (UTRs); (2) mRNAs undergoing "leaky-scanning" for translation initiation; (3) mRNAs regulated by programmed frameshifting; (4) mRNAs with selenocysteine codons; and (5) mRNAs regulated by stop codon readthrough.

Analyses of the gene ontology terms associated with transcripts regulated by NMD reveal that although some fall into functional categories that are over-represented relative to the proportion in the genome, it is the diversity of functional categories that is most striking, indicating that NMD post-transcriptionally regulates a broad range of cellular processes [2,43,53,54].

Strikingly, with few exceptions, the majority of NMD targets in *D. melanogaster* are not orthologues of genes regulated by NMD in *S. cerevisiae* or human cells, indicating that the repertoire of genes targeted by NMD is not conserved [2,43]. This lack of conservation could be explained in part by the different mechanism of PTC-recognition across species. For instance, human transcripts containing introns at least 50 nucleotides downstream of the natural stop are degraded by NMD. These transcripts are not NMD substrates in *S. cerevisiae* and *D. melanogaster*.

One group of orthologous transcripts commonly regulated in the three organisms includes *D. melanogaster* SMG5 and SMG6, human SMG5 and *S. cerevisiae* Ebs1p and Est1p (which share a common domain organization with SMG5–7 [8–10]). The regulation of SMG5 by NMD both in human and *D. melanogaster*, suggests the existence of a conserved feedback mechanism [2,7,43].

In addition to *EBS1* and *EST1*, five genes involved in telomere maintenance are regulated by NMD in *S. cerevisiae*, including *EST2*, *EST3*, *STN1*, *YKu70* and *TEL1* [55,56]. The regulation of genes involved in telomere maintenance by NMD has functional implications as, in *S. cerevisiae*, inhibition of NMD results in telomere shortening and de-repression of silenced telomeric loci [55,56]. Furthermore, ATM (involved in telomere maintenance and DNA repair) is regulated by NMD in *D. melanogaster* [2,43]. Taken together with recent reports implicating human UPF1, SMG5 and SMG6 in telomerase function [9,10], this suggests that the role of NMD in regulating the expression of genes involved in telomere maintenance is conserved.

9. Evolutionary diversification of the biological role of NMD

NMD components are not essential in yeast. Similarly, inhibition of the NMD pathway in *C. elegans* leads to viable worms, albeit with defects in the male bursa and the hermaphrodite vulva [3–5]. Thus, in *S. cerevisiae* and *C. elegans*, NMD is not essential at the cellular level (Table 1). In contrast, UPF1 and UPF2 are essential for larval development in *D. melanogaster* [57]. The NMD pathway is also required at the cellular level, because cells depleted of UPF1 and UPF2 are arrested at the G2/M-phase of the cell cycle, and cell proliferation is impaired by depletion of the additional NMD effectors [43]. Similarly, UPF1 is also required for cell viability in mice as *UPF1* null mice die early in embryonic development and attempts to establish homozygous $UPF1^{-/-}$ ES cells have also failed [58]. Consistently, depletion of UPF1, but not of UPF2, from human cells inhibits cell cycle progression (Table 1) [50]. In *A. thaliana* UPF1 is also an essential gene, and mutant alleles of this gene have pleiotropic effects [47], suggesting that like in animals, the NMD pathway in plants regulates mRNAs with functions in a broad range of cellular processes.

Two mechanisms are likely to contribute to the phenotypic differences observed across species when the NMD pathway is inhibited: one is the lack of conservation of the targets of the pathway; the second is the functional diversification of NMD effectors, which appears to have happened at least in mammals.

10. NMD suppresses or exacerbates the phenotypes associated with specific genetic disorders

About 30% of inherited genetic disorders are caused by nonsense mutations or frameshifts, which generate nonsense codons. Transcripts derived from the mutant alleles are degraded by the NMD pathway, generally leading to a recessive mode of inheritance, as the wild-type protein from the second functional allele is able to compensate, at least in part, for the absence of the nonsense-containing transcript. In some cases, the nonsense transcript escapes NMD, resulting in the production of a truncated protein, and this may lead to a dominant genetic defect. This phenomenon has been shown for the different forms of β-thalassaemia, where some mutations cause a mild, recessive phenotype, while others result in dominantly inherited diseases due to the accumulation and toxic precipitation of insoluble globin chains [59]. Moreover, different phenotypes have been observed for the same mutation in different individuals, suggesting that NMD efficiency is subject to individual variations [59]. Other diseases with dominant and recessive forms due to NMD efficiency are retinitis pigmentosa, von Willebrand disease and factor X deficiency [59].

NMD also protects the organism from the effects of acquired mutations, e.g. during tumour progression. Transcripts of several mutant forms of the tumour suppressor proteins BRCA1, p53 and Wilms tumour (WT1) have been shown to be eliminated by NMD. These mutations would convert the tumour suppressors into dominant-negative oncoproteins if the transcripts were not eliminated by NMD. Indeed, it has been shown that the expression of the corresponding truncated proteins from intronless cDNAs (which are immune to NMD in mammals), leads to increased tumourogenicity, cell survival and resistance to chemotherapy [59]. Hence, NMD protects heterozygous carriers of these mutations from developing cancer, as long as the wild-type allele remains intact.

In the examples described above, NMD acts as a protective surveillance mechanism, and increasing NMD efficiency may be beneficial in these cases. However, in some genetic diseases, where a nonsense mutation targets the mutant transcript for

rapid decay, the loss of the protein product is more deleterious to the cell than the expression of the truncated protein would be. This applies to cases in which the truncated protein does not have dominant-negative effects, and is at least partially functional. Attempts to inhibit NMD in order to restore protein expression levels have been made as a tentative treatment for some genetic diseases (e.g. cystic fibrosis, Duchenne muscular dystrophy, Hurler syndrome and X-linked nephrogenic diabetes insipidus) [59].

Aminoglycoside drugs such as gentamycin, which promote stop codon suppression and thus inhibit NMD, have been used to restore expression of nonsense transcripts. Local administration of gentamycin has yielded some promising results, e.g. in alleviating the symptoms of cystic fibrosis. A major problem associated with the use of this approach is the low efficacy and the secondary effects on normal protein production and function. Therefore, approaches targeting NMD effectors specifically would be more suitable. The possibility that alternative NMD pathways have evolved, and that not all NMD effectors have essential biological functions opens up the potential for approaches targeting specific non-essential NMD components.

11. Concluding remarks

Effectors of the NMD pathway are being explored as potential therapeutic targets in order to regulate the expression of mutated genes in a subset of human genetic disorders. Understanding the mechanism underlying NMD is therefore of critical importance. Studies in model organisms led to the identification of the major players in this pathway, but our understanding of their precise molecular function is still rudimentary. Moreover, information on how the activity of these proteins can be subject to directed regulation is lacking. Furthermore, whether alternative NMD pathways have evolved in higher eukaryotes and whether NMD effectors acquired additional functions need to be established before therapeutic strategies targeting NMD effectors can be envisaged.

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